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Anti-Herpes Simplex Virus Activity of 5-Substituted 2-Pyrimidinone Nucleosides

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Several 5-substituted 2-pyrimidinone 2′-deoxyribonucleoside (PdR) analogs were examined for their anti-herpes simplex virus (HSV) activity in cell culture. The order of potency of their antiviral activities against HSV type 1 (HSV-1) and HSV-2 was iodo PdR ≈ ethynyl PdR ≈ propynyl PdR. The antiviral action of iodo PdR is dependent on the ability of HSV to induce virus-specified thymidine kinase in infected cells. Several HSV-1 variants with altered thymidine kinase changed their sensitivity to iodo PdR, whereas HSV-1 variants with altered DNA polymerase were as sensitive as the parental virus to iodo PdR. Continuous presence of iodo PdR for more than one virus replication cycle was required for optimal antiviral activity. Iodo PdR (100 μM) had no activity against Epstein-Barr virus DNA replication in P3HR-1 cells. With an oral, an intraperitoneal, or a subcutaneous route of injection, iodo PdR administered twice a day for 2.5 days could prevent the death of mice infected with HSV-2. This in vivo activity is unlikely to be related to the potential conversion of iodo PdR to iododeoxyuridine, since iodo PdR is not a substrate of xanthine oxidase.

Herpes simplex virus types 1 and 2 (HSV-1 and -2) are recognized as inducers of type-specific thymidine kinase (TK) in virus-infected cells. These viral TKs have a broader spectrum of substrate specificity than host TK (5, 7). The development of compounds based on the unique properties of HSV TK has already been proposed (6), and several nucleoside analogs were developed and used as anti-HSV compounds in the clinic. Although virus TK is involved in the selectivity of these anti-HSV nucleosides, it should be noted that the metabolisms and mechanisms of action could be quite different. The synthesis of new anti-HSV nucleoside analogs based on viral TK with different mechanisms of action is continually being pursued by many laboratories. This communication reports on the anti-HSV activity of a newly synthesized 2-pyrimidinone nucleoside (Fig. 1) in both cell culture and mice. A preliminary report on the activity of 5-iodo-2-pyrimidinone 2′-deoxyribonucleoside (IPdR) was published previously (16).

MATERIALS AND METHODS

Sodium *n*-butyrate, 12-*O*-tetradecanoylphorbol 13-acetate, xanthine, and *N,N*-dimethylaniline were purchased from Sigma Chemical Co., St. Louis, Mo. [α-^{32}P]dCTP was purchased from ICN Pharmaceuticals Inc., Irvine, Calif. Nitrocellulose was from Schleicher & Schuell, Inc., Keene, N.H. The Random Primed DNA Labeling Kit, 3-methyl-2-benzothiazolinone hydrazone hydrochloride, peroxidase, and xanthine oxidase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The 5-substituted 2-pyrimidinone 2′-deoxyribonucleoside (PdR) analogs, iodo PdR (IPdR), ethynyl PdR (EPdR), propynyl PdR (PPdR), and methyl PdR (MPdR), were made in the laboratory of Thomas Bardos, Department of Medicinal Chemistry, State University of New York, Buffalo (16).

Animal studies. Swiss Webster female mice (Hilltop Laboratories, Chatsworth, Calif.), 6 to 8 weeks old and weighing approximately 20 g, were infected intraperitoneally on day 0 with 5×10^5 PFU or 1×10^6 PFU of HSV-2 (strain 333). The drugs were administered intraperitoneally, subcutaneously, or orally once a day for 5 days or twice daily (at 12-h intervals) for 2.5 days beginning 24 or 72 h postinfection. Mean body weight determinations of drug-treated and control animals were made during the treatment period as an indicator of sublethal toxicity. Deaths were recorded through day 45.

Cells. All cells were cultured in RPMI 1640 medium supplemented with either 10% (P3HR-1) or 5% (HeLa and Vero) fetal bovine serum and 100 μg of kanamycin per ml. The cells were maintained in a humidified atmosphere at 5% CO_2 at 37°C.

Virus. Phosphonoformic acid (PFA)-resistant HSV-1 mutants were developed as previously described (2) in the laboratory of Y.-C. Cheng (Department of Pharmacology, University of North Carolina, Chapel Hill). HSV-1 variants with an altered TK were a gift from H. J. Field (Department of Pathology, Cambridge University, Cambridge, United Kingdom) and were developed as previously described (11). The HSV-1 variant MDK was a gift from S. Kit, College of Medicine, Baylor University, Houston, Tex. (10).

All the viruses (HSV-2 strain 333 and all HSV-1 strains) were maintained as previously described (8), with the exception that Vero cells were used instead of CV-1 cells and the multiplicity of infection was 0.01 PFU per cell.

In vitro experiments. For HSV studies, a monolayer of HeLa S_3 cells was infected with HSV at a multiplicity of 3 PFU per cell. After 1 h of adsorption, the inoculum was removed, and medium was added with or without the indicated concentrations of IPdR (see legend to Fig. 2). The total incubation time was 24 h, unless otherwise indicated. To determine the reversibility of the antiviral activity of IPdR, the drug was removed 12 h postinfection and replaced with fresh drug-free medium. The cells were incubated for a total of 36 h postinfection. At the end of incubation, the flasks were stored at −70°C until virus titration. The proce-

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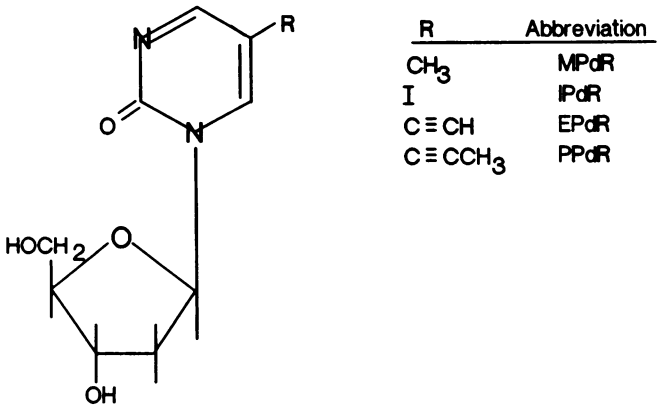

FIG. 1. Structures of 5-substituted 2-pyrimidinone analogs. Abbreviations: MPdR, 5-methyl-2-pyrimidinone; IPdR, 5-iodo-2-pyrimidinone; EPdR, 5-ethynyl-2-pyrimidinone; and PPdR, 5-propynyl-2-pyrimidinone.

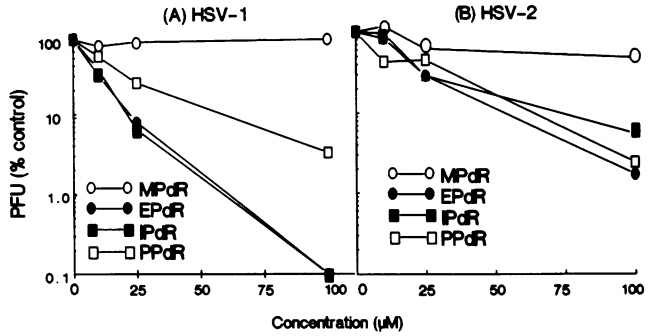

FIG. 2. Antiviral activity of 2-pyrimidinone nucleoside analogs in vitro. HeLa S_3 cells infected with either HSV-1 or HSV-2 were treated with 10, 25, or 100 μM IPdR for 24 h. Virus production (in PFU per milliliter) was assessed by a virus yield assay and is expressed as a percentage of the no-drug control. The determinations represent the average of duplicate samples from the same experiment.

dure for virus titration was as described previously (8), with the exception that Vero cells were used instead of CV-1 cells.

The induction of Epstein-Barr virus (EBV) was performed as previously described (13), except with P3HR-1 cells instead of Raji cells. Briefly, P3HR-1 cells were plated at a density of 5×10^5 cells per ml and incubated for 48 h at 37°C in the presence of 4 mM sodium *n*-butyrate and 50 nM 12-*O*-tetradecanoylphorbol 13-acetate with or without 100 μM IPdR. EBV DNA synthesis was analyzed by in situ discontinuous agarose gel electrophoresis as previously described (13), with the exception that only two different percentages of agarose were used. The body of the gel was prepared by using 0.8% agarose, and the well area was prepared by using 0.4% agarose. The transfer of DNA to a nitrocellulose filter was accomplished by use of a vacuum blot apparatus (American Bionetics, Inc., Hayward, Calif.) according to the instructions of the manufacturer. Hybridization was then performed with the *Eco*RI C fragment of EBV labeled with [^{32}P]dCTP (labeling was done with a Random Primed DNA Labeling Kit [Boehringer Mannheim] according to the instructions of the manufacturer).

Xanthine oxidase assay. A colorimetric method for the assay of xanthine oxidase activity based on the production of hydrogen peroxide (H_2O_2) was used to determine the ability of xanthine oxidase to oxidize IPdR. The assay was performed as described previously (18). Briefly, various concentrations of xanthine or IPdR were incubated with 0.1 M phosphate buffer (pH 8.5) containing 1 mM sodium azide for 5 min at 37°C. Xanthine oxidase was then added to the substrate mixture, and incubation continued at 37°C. The oxidation reaction, which produces an oxidized product and H_2O_2, was stopped at the indicated times (see Fig. 4) with addition of 0.2 M citric acid. A color reagent (9.6 mg of 3-methyl-2-benzothiazolinone hydrazone hydrochloride, 0.3 ml of *N,N*-dimethylaniline, and 400 U of peroxidase dissolved in 100 ml of 0.2 M citrate-phosphate buffer [pH 3.5]) was then added to the samples. A coupling reaction between 3-methyl-2-benzothiazolinone hydrazone hydrochloride and *N,N*-dimethylaniline with H_2O_2 took place, producing an indamine dye and water. The production of the dye was then read at A_{600}.

RESULTS

Several of the pyrimidinone analogs were screened for antiviral activity in cell culture. The order of potency of the analogs was IPdR = EPdR > PPdR > MPdR for HSV-1 and IPdR ≈ EPdR ≈ PPdR > MPdR for HSV-2 (Fig. 2). IPdR showed significant anti-HSV activity with 50% inhibitory concentrations of 13 and 19 μM against HSV-1 and HSV-2, respectively. In addition, the cytotoxicity of IPdR in uninfected HeLa S_3 cells was 11% at 200 μM.

The antiviral activity of IPdR against various HSV-1 mutants was also determined. The results are shown in Table 1. IPdR had a much reduced antiviral effect against HSV-1 variants that are either deficient in or have an altered HSV-specified TK. However, the antiviral activity of IPdR was not reduced in variants which are PFA and 9-(2-hydroxyethoxymethyl)guanine (ACV) resistant by means of an altered DNA polymerase. This result was not unexpected, since the antiviral activity of IPdR is dependent on the HSV-specified TK.

The ability of HSV-2 replication (activity) to recover after removal of IPdR from the cell cultures was examined and is shown in Fig. 3. While the continued presence of IPdR was not required for antiviral activity, the optimal antiviral activity was obtained when IPdR was present for more than one virus replication cycle. By comparison, the antiviral action of (*E*)-5-(2-bromovinyl)-2-deoxyuridine (BVDU)

TABLE 1. Antiviral activity of IPdR against HSV-1 variants

HSV-1[a] strain	Description of strain	% PFU[b]
SCS	Parental strain	1.2
S1	TK altered; BVDU and ACV resistant	47
B3	TK altered; BVDU resistant	95
Tr7	TK altered; ACV resistant	19
MDK (2006)	TK deficient	56
KOS	Parental strain	0.4
PFA^{r}1a	DNA POL[c] altered; PFA and ACV resistant	0.4
PFA^{r}3b	DNA POL altered; PFA and ACV resistant	0.5
PFA^{r}5a	DNA POL altered; PFA and ACV resistant	0.2

[a] For the origin of virus strains, see Materials and Methods.
[b] The sensitivity of the viruses in the presence of 50 μM IPdR for 24 h was determined by a virus yield assay. For virus yield assay details, see reference 8. Data are expressed in percent PFU relative to a no-drug control.
[c] DNA POL, DNA polymerase.

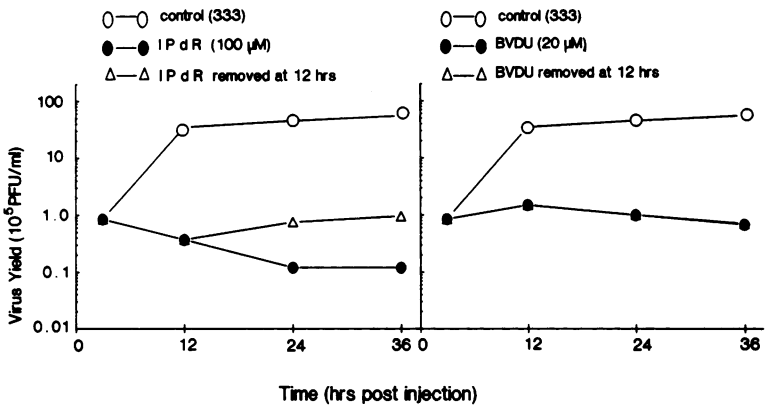

FIG. 3. Recovery of HSV-2 activity after removal of IPdR from cell culture. HeLa S_3 cells infected with HSV-2 were incubated with or without 100 μM IPdR. At 12 h postinfection, IPdR was removed from one set of infected cells and replaced with medium without IPdR. Total incubation time was 36 h. Virus production (PFU per milliliter) was assessed by a virus yield assay. The determinations represent the average of duplicate samples from the same experiment. BVDU, (*E*)-5-(2-Bromovinyl)-2-deoxyuridine.

against HSV-2 was not reversible after removal. The irreversible antiviral action may be due to the incorporation of BVDU into the viral DNA (1, 12).

An in situ, discontinuous agarose gel was used to examine the effect of IPdR on the synthesis of EBV DNA (for details see Materials and Methods). The induction of EBV-specified DNA was not detectably affected in the presence of 100 μM IPdR (results not shown). This lack of activity against EBV may be due to the absence of phosphorylation of IPdR in EBV-containing cells.

The antiviral activity of IPdR was also studied in vivo (Table 2). A dose-dependent response to orally administered IPdR was observed. Orally administered IPdR (100 mg/kg) was comparable to orally administered 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) (40 mg/kg) (for discussion of antiviral activity of DHPG see reference 17). However, regardless of the route of administration, IPdR could significantly increase the life span of mice infected with HSV-2 when given twice a day for 2.5 days. Also, the same potent antiviral effect of IPdR was observed when IPdR was administered to the animals at the onset or well after the initiation of HSV-2 infection. In addition, IPdR was still effective when the inoculum dose of HSV-2 was increased to 10^6 PFU. No significant toxicity as determined by weight loss was observed for any of the administered compounds.

The potent antiviral activity in vivo and the pyrimidinone structure of IPdR led us to study the possible oxidation of IPdR to iododeoxyuridine (IUdR), another potent antiviral nucleoside analog (14). A likely enzyme for this oxidation reaction is xanthine oxidase. Xanthine oxidase is known to have a low substrate specificity and is able to oxidize xanthine, purines, and aldehydes (3, 4, 15). To determine if IPdR could be oxidized to IUdR by xanthine oxidase, 1 mM or 300 μM IPdR was added as a substrate to a colorimetric xanthine oxidase assay. Xanthine oxidase was added to a concentration of 10×10^{-3} or 100×10^{-3} U per assay. The oxidation of IPdR was assayed over a period of 2 h (Fig. 4). The production of H_2O_2 from the oxidation of IPdR could not be detected even after 2 h, whereas in the same experiment, the oxidation of xanthine could be detected very soon after the addition of xanthine oxidase and the oxidation reaction was completed by 3 min. Even with a fivefold-lower concentration of xanthine oxidase, the oxidation of xanthine was completed within 10 min.

DISCUSSION

The 5-substituted 2-pyrimidinone 2′-deoxyribonucleoside analogs, which share a common structure with thymidine

TABLE 2. Antiviral activity of IPdR in HSV-2 (333)-infected mice[a]

Drug (mg/kg)	Route[b]	Schedule[c]	No. of survivors/ total (%)[d]	Mean survival time (days) (± SD)[e]	% ILS[f]
Control	i.p.	1 × 5	1/10 (10)	9.6 ± 1.1	
EPdR (100)	i.p.	1 × 5	1/5 (20)	9.0 ± 0.8	94
IPdR (100)	i.p.	1 × 5	7/10 (70)	12.0 ± 4.4	129
Control	s.c.	1 × 5	0/5 (0)	9.4 ± 1.1	
IPdR (100)	s.c.	1 × 5	2/5 (40)	12.3 ± 1.2	131
Control	i.p.	2 × 2.5	0/10 (0)	9.3 ± 1.1	
IPdR (10)	i.p.	2 × 2.5	0/5 (0)	12.8 ± 3.6	133
IPdR (40)	i.p.	2 × 2.5	2/5 (40)	10.0 ± 1.0	108
IPdR (100)	i.p.	2 × 2.5	6/10 (60)	12.5 ± 1.3	135
Control	Oral	2 × 2.5	4/20 (20)	10.6 ± 2.1	
IPdR (10)	Oral	2 × 2.5	0/5 (0)	12.8 ± 3.6	123
IPdR (40)	Oral	2 × 2.5	2/5 (40)	10.0 ± 1.0	94
IPdR (100)	Oral	2 × 2.5	20/20 (100)	>45	
DHPG (40)	Oral	2 × 2.5	20/20 (100)	>45	
Control[g]	Oral	2 × 2.5	0/5 (0)	10.0 ± 1.2	
IPdR (100)	Oral	2 × 2.5	4/5 (80)	14.0	140
DHPG (40)	Oral	2 × 2.5	2/5 (40)	20.0 ± 7.0	200
Control[h]	Oral	2 × 2.5	0/5 (0)	9.8 ± 1.3	
IPdR (100)	Oral	2 × 2.5	5/5 (100)	>45	
DHPG (40)	Oral	2 × 2.5	5/5 (100)	>45	

[a] Mice were infected intraperitoneally with 5×10^5 PFU of HSV-2 (333).
[b] i.p., Intraperitoneal; s.c., subcutaneous.
[c] The number of daily doses of drug times the number of days treatment was given.
[d] Percent survival is the number of mice alive at 45 days over the total number per group.
[e] The average day of death of the mice in the experimental group.
[f] ILS, Increased life span of treated mice (increased over mean life span of untreated mice) in percent, excluding 45-day survivors.
[g] Mice in this experiment were inoculated with 1×10^6 PFU instead of 5×10^5 PFU.
[h] Treatment was begun 72 h after implant instead of the usual 24 h.

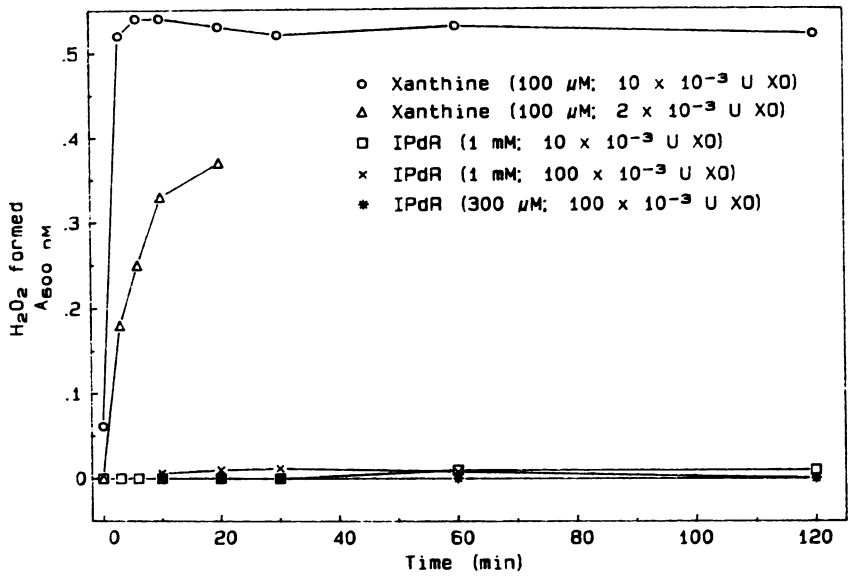

FIG. 4. Oxidation of IPdR by xanthine oxidase. IPdR (1 mM or 300 μM) or xanthine (100 μM) was incubated with xanthine oxidase. Hydrogen peroxide (H_2O_2) was measured over the course of 120 min by the procedure described in Materials and Methods.

and deoxycytidine, were strategically developed as anti-HSV agents (16). The strategy of development was based on the hypothesis that nucleosides without an amino group or oxygen at position 4 could be utilized as substrates by viral TK, but not by cellular nucleoside kinases. Therefore, pyrimidinone analogs could be selectively phosphorylated by HSV-specified TK. Several of the pyrimidinone analogs were screened for antiviral activity in cell culture. Even though EPdR had antiviral activity equal to or better than that of IPdR in vitro, IPdR was chosen for further study, since IPdR in vivo is much more active than EPdR (Table 2). The dependence of the antiviral activity of IPdR on the HSV-specified TK is evident by its reduced activity in HSV-1 variants with modified TK but not in variants with altered DNA polymerase.

The antiviral activity of IPdR in vivo can be maintained when the agent is given orally but probably is not maintained as a result of the agent being metabolized to IUdR by xanthine oxidase. The sensitivity of the xanthine oxidase assay is such that if in 120 min 4,000-fold less of the end product (H_2O_2) was formed, it would still be detectable. Thus, the sensitivity of the assay should allow the detection of IPdR product conversion if it can be utilized by xanthine oxidase as a substrate. Although we cannot rule out that there are other enzymes capable of converting IPdR to IUdR in mice, it can be concluded that IPdR is not a substrate of xanthine oxidase. In addition, IUdR has been shown to be toxic (14), whereas IPdR has no toxicity at effective dosages in vivo. In view of that, it is unlikely that the observed anti-HSV-2 activity in mice is the result of conversion of IPdR to IUdR.

In conclusion, we have shown that the pyrimidinone nucleoside analog IPdR has very potent antiviral activity against HSV-1 and HSV-2 in vitro and against HSV-2 in vivo. The antiviral selectivity and activity of IPdR is dependent on the HSV-specified TK. In view of the substrate specificity of nucleoside monophosphate kinases and DNA polymerase, this compound may not be easily incorporated into DNA, which is the key mechanism of action of other viral-TK-dependent antiviral compounds (9, 17). It is conceivable that the active metabolite may be the monophosphate nucleoside and that the mechanism of action is quite different from those of other antiviral compounds. The metabolism and mechanism of action of IPdR are being investigated.

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